

Nutritional requirements and penicillin susceptibilities of gonococci from pharyngeal and anogenital sites

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SUMMARY *Neisseria gonorrhoeae* was isolated from 5.9% of oropharyngeal specimens obtained from patients attending a clinic for sexually transmitted diseases. Oropharyngeal isolates from 69 patients and anogenital isolates from 97 other patients attending the same clinic were compared. Many of the gonococci could be differentiated by the compounds required for growth in chemically defined media or by differences in the minimum inhibitory concentration (MIC) of penicillin G. Strains with requirements for either proline (Pro⁻) or arginine (Arg⁻) or for none of the compounds that are used for differentiation (zero phenotype) were more common in the oropharynx (91.3% of patients) than in anogenital sites (73.2% of patients). On the other hand, gonococci with multiple requirements that include arginine, hypoxanthine, and uracil (AHU strains) were present in oropharyngeal specimens from only three patients (4.4%), but were isolated from anogenital specimens from 18 patients (18.6%). A high susceptibility to penicillin characterised the AHU strains from all sites, as others have reported. The penicillin MIC ranged from 0.003-0.72 µg/ml for strains with Pro⁻, Arg⁻, and zero phenotypes. However, a penicillin MIC ≥ 0.42 µg/ml was found for 17.6% of oropharyngeal isolates of these types, but for only 4.1% of Pro⁻, Arg⁻, and zero isolates from anogenital sites. None of these moderately resistant strains produced β -lactamase. Our findings indicate that gonococci differ in their ability to colonise the oropharynx successfully.

Introduction

Infections of the oropharynx caused by *Neisseria gonorrhoeae* were seldom recognised until Fiumara *et al.* (1967) described gonococcal pharyngitis in three homosexual men, two of whom were without clinical or bacteriological signs of urethritis or proctitis. Pharyngeal or tonsillar infections are now recognised as being relatively common and often asymptomatic. In studies of large numbers of consecutive patients with gonorrhoea who were examined in Seattle (Wiesner *et al.*, 1973) and in Copenhagen (Bro-Jørgensen and Jensen, 1973) the incidence of pharyngeal infection with gonococci was, respectively, 21 and 25% in homosexual men, 3 and 7% in heterosexual men, and 10% in women of both study groups.

Much remains to be learned about the biology of gonococci and the various differences between the strains associated with uncomplicated gonorrhoea

and with disseminated gonococcal infections. One of the newer ways to characterise gonococci is to determine the compounds that they require for growth in chemically defined media. Most strains of *N. gonorrhoeae* are able to multiply in a complete medium, but many strains fail to grow in the absence of particular amino-acids, nucleic acid bases, or vitamins (Catlin, 1973). These nutritional requirements were exploited as a means of differentiating between gonococci by a system of auxotyping (Carifo and Catlin, 1973). Differences have been found between the percentages of various gonococcal auxotypes implicated in uncomplicated anogenital infection and in disseminated gonococcal infection (Knapp and Holmes, 1975; Morello *et al.*, 1976; Eisenstein *et al.*, 1977). We have investigated the prevalence of *N. gonorrhoeae* in pharyngeal specimens and compared the auxotypes and penicillin susceptibilities of isolates from pharyngeal and anogenital specimens.

Materials and methods

Specimens for culture of gonococci were obtained

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during routine examinations of patients attending the Social Hygiene Clinic of the Milwaukee Health Department. Specimens were collected with cotton swabs and were immediately streaked on to modified Thayer-Martin medium (containing vancomycin, colistin, nystatin, and trimethoprim). Colonies of typical cocci were picked after incubation at 35°C for 48 hours in a candle flame-extinction jar. Oxidase-positive, Gram-negative diplococci from all specimens were identified as *N. gonorrhoeae* by their production of acid from dextrose, but not from maltose, sucrose, or lactose. The media and procedures used for these tests and the characteristics of *Neisseria meningitidis* and *Neisseria lactamica* have been described (Catlin, 1974).

Gonococci were stored at -60°C as dense suspensions of bacteria in 1 ml of trypticase soy broth (BBL) supplemented with 20% by volume of glycerol. In preparation for determinations of penicillin susceptibility or auxotype, frozen cultures were thawed at 23°C and cultivated for between 16 and 18 hours on antibiotic-free GC medium base with supplements.

The chemically defined media used for auxotyping were described earlier (Carifo and Catlin, 1973; Catlin, 1973) and the technical procedures, including inoculation by the Steers replicating apparatus, have been given in greater detail recently (Catlin, 1977). Soluble starch (0.1%) was added to all *Neisseria* defined agar media (NEDA) and all amino-acids used were L isomers. The set of media included the complete NEDA, and NEDA with the omission of cysteine and cystine. All strains of *N. gonorrhoeae* examined in this study grew on NEDA and none grew on NEDA lacking cysteine and cystine, which provided a valuable confirmatory test for *N. gonorrhoeae*. The differential compounds singly omitted from NEDA included proline, arginine, methionine, histidine, lysine, leucine, hypoxanthine, and uracil. One medium lacked the mixture of vitamins and cofactors, -V medium (Carifo and Catlin, 1973). Gonococci that failed to grow on -V medium were tested for growth on three additional media: -V with single omissions of thiamine, thiamine pyrophosphate, or biotin. Gonococci that failed to grow on the arginine-free medium were examined additionally for ability to grow on NEDA that contained ornithine in place of arginine. Standardised suspensions of gonococci prepared in buffered salts solution were deposited (10^3 - 10^4 colony-forming units) by the Steers inoculating device on the surfaces of auxotyping media. The requirement for a differential compound was indicated by the absence of macroscopic growth on a medium lacking that compound after incubation for 48 hours in air plus 8% CO₂. A haze of microcolonies

or a single macrocolony was disregarded.

Tests of gonococcal susceptibility to benzyl penicillin (penicillin G potassium, 1580 international units per mg, supplied by Abbott Laboratories, North Chicago, Illinois) were made using various concentrations of penicillin incorporated in Proteose Peptone No. 3 agar (Difco) with haemoglobin and supplements prepared according to a standard procedure (Public Health Service, 1963). Suspensions of patient isolates and control bacteria of known susceptibility were prepared and standardised as described by J. D. Thayer, J. E. Martin, Jr, and A. Lester (Venereal Disease Branch, Center for Disease Control, Atlanta, Georgia, personal communication, 1967). A Steers inoculating device was used to deposit $1-2 \times 10^3$ colony-forming units on the media and the plates were incubated at 35°C in candle flame-extinction jars for 48 hours. The minimum inhibitory concentration (MIC) of penicillin was taken as the lowest concentration that inhibited growth, disregarding a faint haze or a single colony.

The test for gonococcal penicillinase (β -lactamase) used an iodometric method described previously (Catlin, 1975).

Results

PREVALENCE OF NEISSERIA

To compare the percentages of gonococcal isolates from the oropharynx, cervix, and anal canal, specimens from these three sites were obtained from each of 370 women who attended the Milwaukee Health Department Social Hygiene Clinic in 1973. Gonococci were cultured from at least one specimen from 93 (25.1%) of these patients; 16 positive pharyngeal cultures were obtained, including five which provided the only evidence of gonorrhoea. Therefore, since 1973 oropharyngeal specimens for culture of gonococci have been obtained from all clinic patients who complain of a sore throat or admit to oral-genital contacts.

N. gonorrhoeae was isolated from 170 (5.9%) of the 2879 oropharyngeal specimens obtained during the four-year period, 1973-76. During the same time, gonococci were recovered from 19.6% of 22 458 cervical specimens, 4.7% of 6966 swabs from the anal canal, and 6.0% of 15 682 specimens from the male urethra.

N. meningitidis was found in 25.8% of the same oropharyngeal cultures examined during the four-year period and, in addition, 30 cultures (1.0%) yielded *N. lactamica*. The recognition that *N. gonorrhoeae* colonises the oropharynx has made investigators increasingly alert to the inverse situation—namely, that pharyngeal flora may colonise urogenital sites (Givan and Keyl, 1974). In our

series of cultures typical strains of *N. meningitidis* were isolated from 10 specimens from the male urethra and three cervical specimens as well as from 30 rectal cultures.

GONOCOCCAL AUXOTYPES

The auxotypes and penicillin susceptibilities were determined for 89 gonococci isolated from oropharyngeal specimens from 69 patients. Two of these isolates were collected in 1972 and the remainder during the three-year period from June 1973 to May 1976. The results, presented in the Table, show that 91.3% of these patients harboured *N. gonorrhoeae* with nutritional requirements for either proline (Pro⁻) or arginine (Arg⁻), or for none of the compounds that are used to differentiate between auxotypes (zero phenotype). Gonococci with multiple requirements which include arginine, hypoxanthine, and uracil (AHU strains) were isolated from oropharyngeal specimens of only three (4.4%) of these patients.

The auxotypes of gonococci isolated from urogenital or rectal specimens of patients attending the same Social Hygiene Clinic have been examined in two studies. The Table gives the auxotypes of 97 anogenital isolates from patients who attended in July and August 1975. The phenotypes zero, Pro⁻, and Arg⁻ accounted for 73.2% of these strains, whereas 18.6% were AHU strains. The earlier study (Carifo and Catlin, 1973) of 251 anogenital isolates from patients attending the clinic during the period August to October 1972 found that 76.9% of these gonococci were zero, Pro⁻, or Arg⁻ types and 17.5% were AHU strains.

Most of the oropharyngeal isolates were collected during the time spanned by the two studies of anogenital isolates. Of the 38 gonococci isolated from oropharyngeal cultures in 1973, 25 were saved at random for auxotyping and none was an AHU strain. The three patients from whom AHU strains were isolated each attended during different years (1974-76). Some statistical uncertainty is inherent in the fact that oropharyngeal isolates from only 69 patients were typed. However, the finding that AHU strains were recovered from pharyngeal specimens from only 4.4% of these 69 patients whereas 17.8% of AHU strains were recovered from anogenital specimens (348 patients) suggests that biological differences between the gonococci affect their ability to colonise different tissues of the patient.

PENICILLIN SUSCEPTIBILITIES

A high susceptibility to penicillin is typical of AHU strains (Knapp and Holmes, 1975; Morello *et al.*, 1976; Eisenstein *et al.*, 1977). Similarly, the MIC of benzylpenicillin was ≤ 0.006 $\mu\text{g/ml}$ for 16 of the

AHU strains in the Table and the remaining five were inhibited by the next higher concentration of penicillin tested. The zero, Pro⁻, and Arg⁻ strains, on the other hand, were heterogeneous in their responses to penicillin; for the majority the penicillin MIC was ≥ 0.06 $\mu\text{g/ml}$. Anogenital specimens from only four patients (4.1%) yielded gonococci which had a penicillin MIC ≥ 0.42 $\mu\text{g/ml}$. In contrast, an MIC ≥ 0.42 $\mu\text{g/ml}$ was recorded for gonococci isolated from the oropharynx of 12 patients (17.4%). Larger percentages of partially resistant gonococci were present also in pharyngeal or tonsillar sites of patients described by Ødegaard and Gundersen (1973) and by Hallqvist and Lindgren (1975).

REPEATED ISOLATIONS

Determinations of gonococcal auxotype and penicillin MIC provide data sufficient, in some cases, to differentiate between treatment failure and reinfection from a new source. For example, different gonococcal strains were isolated from oropharyngeal specimens taken from one man on two occasions and each is recorded separately in the Table. One strain was Arg⁻ and characterised by a penicillin MIC of 0.06 $\mu\text{g/ml}$, whereas the second was Pro⁻ and more resistant (MIC 0.3 $\mu\text{g penicillin/ml}$). Oropharyngeal specimens from 13 other patients (10 male and three female) each yielded gonococci on two or more—up to five—occasions. The same auxotype and penicillin MIC characterised the several isolates from a patient (Table). Two of these patients were treated in the clinic only on the second visit, specimens having been taken both times. The interval between cultures of these untreated patients documented the persistence of infection with an Arg⁻ strain (penicillin MIC 0.12 $\mu\text{g/ml}$) for at least 29 days and an AHU strain for at least 18 days. Gonococcal infections which persisted in the pharynx for between one and four months have been reported (Bro-Jørgensen and Jensen, 1973; Wiesner *et al.*, 1973).

Gonococci were recovered from oropharyngeal specimens from one male and one female patient who returned for follow-up examinations on the seventh day after receiving intramuscular injections of 4.8 million units of procaine penicillin G (the man additionally received 1.0 g of probenecid orally). An Arg⁻ strain of *N. gonorrhoeae* with penicillin MIC of 0.06 $\mu\text{g/ml}$ was isolated from the initial and test-of-cure cultures from the man; both specimens from the woman yielded Pro⁻ gonococci that were relatively resistant to penicillin (MIC 0.6 $\mu\text{g/ml}$). Intervals longer than one week between penicillin treatment and the follow-up visits of six other patients increased the chance that reinfection from the same consort, rather than treatment failure,

might account for the presence in the test-of-cure specimen of gonococci having the same auxotype and penicillin MIC as the initial isolate.

Three patients received intramuscular injections of spectinomycin (2.0 g for each of the two men and 4.0 g for one woman). One man was culture-negative after seven days; his four previous oropharyngeal specimens had yielded a Pro⁻ gonococcus with a penicillin MIC of 0.6 µg/ml. Pharyngeal specimens taken from the other two patients seven days and 15 days after treatment yielded, respectively, an AHU strain (penicillin MIC 0.006 µg/ml) and a Pro⁻ strain (penicillin MIC 0.24 µg/ml) which corresponded to the pre-treatment isolates (Table).

PENICILLINASE DETERMINATIONS

Penicillin and ampicillin fail to eliminate infections caused by penicillinase-producing gonococci (Percival *et al.*, 1976; McCormack, 1977). Therefore, those strains having a penicillin MIC >0.3 µg/ml which were isolated from 24 patients (Table) were examined for possible β-lactamase activity. No inactivation of penicillin G was found, except in control tests of penicillinase-producing *N. gonorrhoeae* (strains generously provided by Clyde Thornberry, Center for Disease Control, Atlanta, Georgia). Further-

more, since January 1977 the Bureau of Laboratories has performed iodometric tests for β-lactamase on all confirmed cultures of *N. gonorrhoeae*. No penicillinase-producing gonococci have been detected among >1000 isolates examined during the intervening four months.

Discussion

The nutritional requirements of *N. gonorrhoeae* provide a means of subdividing isolates into more than 35 auxotypes which are of value for epidemiological studies (Catlin, 1977). Furthermore, the defects of biosynthetic pathways revealed by the nutritional requirements may have diverse biological consequences (Catlin, 1976). AHU strains as a group (that is, gonococci with multiple requirements which include arginine, hypoxanthine, and uracil) appear to possess special characteristics. Knapp and Holmes (1975) and Morello *et al.* (1976) independently recognised that a large percentage of disseminated gonococcal infections are caused by AHU strains and that all AHU strains are highly susceptible to penicillin. These findings were confirmed by Schoolnik *et al.* (1976), Brooks *et al.* (1976), and Eisenstein *et al.* (1977) who further demonstrated that AHU strains are resistant to the bactericidal

Table Nutritional requirements and penicillin G minimum inhibitory concentration (MIC) for *Neisseria gonorrhoeae* isolated from oropharyngeal specimens and anogenital specimens

Phenotype*	No. of patients	No. of gonococcal isolates	No. of strains with penicillin MIC (µg/ml) at indicated level										
			0.003	0.006	0.03	0.06	0.12	0.18	0.24	0.3	0.42	0.6	0.72
Oropharyngeal	69	89											
Zero	23	25	0	2	4	3†	5	3	2	3	1†	0	0
Pro ⁻	23	34	0	0	3	2	6†	0	2†	2	2†	5†	1
Arg ⁻	17	22	0	0	1	4†	3†	3†	3	0	3	0	0
Arg ⁻ , Hyx ⁻ , Ura ⁻ , Leu ⁻	1	1	0	0	1	0	0	0	0	0	0	0	0
Arg ⁻ , Hyx ⁻ , Ura ⁻ , Leu ⁻ , plus other markers‡	2	4	0	2†	0	0	0	0	0	0	0	0	0
Met ⁻	1	1	0	0	0	1	0	0	0	0	0	0	0
Arg ⁻ , Thi ⁻	1	1	0	0	0	0	1	0	0	0	0	0	0
Pro ⁻ , Arg ⁻ , His ⁻ , Bio ⁻	1	1	0	0	1	0	0	0	0	0	0	0	0
Anogenital	97	97											
Zero	23	23	1	3	7	6	2	2	1	0	1	0	0
Pro ⁻	29	29	0	1	12	8	4	0	1	0	1	2	0
Arg ⁻	19	19	0	2	1	2	6	1	4	3	0	0	0
Pro ⁻ , Arg ⁻	3	3	0	0	1	0	2	0	0	0	0	0	0
Arg ⁻ , Hyx ⁻ , Ura ⁻	12	12	1	8	3	0	0	0	0	0	0	0	0
Arg ⁻ , Hyx ⁻ , Ura ⁻ , plus other requirements§	6	6	0	5	1	0	0	0	0	0	0	0	0
Other¶	5	5	1	0	2	2	0	0	0	0	0	0	0

*Abbreviations for nutritional requirements: zero, no differential compound required; Pro⁻, proline; Arg⁻, arginine; Hyx⁻, hypoxanthine; Ura⁻, uracil; Leu⁻, leucine; Met⁻, methionine; Thi⁻, thiamine or thiamine pyrophosphate; His⁻, histidine; Bio⁻, biotin; Thp⁻, thiamine pyrophosphate (not satisfied by thiamine).

†Two or more similar isolates were cultured from pharyngeal specimens taken from one or more of the patients at different times.

‡Pro⁻ and Met⁻ were additional requirements for one strain; both strains were unable to use ornithine in place of arginine.

§Leu⁻ (two strains), Met⁻ (one strain), Thp⁻ (three strains); two of the strains were unable to use ornithine in place of arginine.

¶Single requirements: His⁻ (one strain), Thp⁻ (one strain), Hyx⁻ (two strains); one strain was Pro⁻ Hyx⁻.

action of human serum. In addition, AHU strains tend to be more susceptible than other gonococci to the toxic action of free fatty acids that contaminate some preparations of agar (Knapp and Holmes, 1975) and to imbalances of branched-chain amino-acids in defined media (Morello *et al.*, 1976).

The data in the Table indicate that colonisation of the oropharynx occurs less often with AHU strains than with gonococci that have a greater biosynthetic competence, in particular strains with zero, Pro⁻, and Arg⁻ phenotypes. AHU strains were responsible for 18% of anogenital infections, but for only 4.4% of pharyngeal infections. On the other hand, zero, Pro⁻, and Arg⁻ strains together accounted for 91% of pharyngeal infections (Table), but for only 76% of the anogenital infections of 348 patients examined in two studies, see Table (Carifo and Catlin, 1973). Inasmuch as these findings relate to patients attending the same clinic, it is likely that the percentage of AHU strains actually recovered from the oropharynx is disproportionately smaller than the percentage initially introduced. Apparently, differences between gonococci affect their ability to persist in the diverse microenvironments of the oropharynx, cervix, urethra, and anal canal. Further studies are needed which compare the auxotypes and various other characteristics of gonococci isolated from these various sites of localised infections as well as from disseminated infections.

Strains of *N. gonorrhoeae* that produce β -lactamase have been incriminated recently in gonorrhoea of some patients who have been treated unsuccessfully with penicillin (Percival *et al.*, 1976; McCormack, 1977). Except for their ability to inactivate penicillins, these strains appear to be typical gonococci, being transmitted freely when introduced into a community and responsible for complications at usual frequencies (Percival *et al.*, 1976). Spectinomycin is a recommended alternative to penicillin (Percival *et al.*, 1976; McCormack, 1977). However, spectinomycin is relatively ineffective for treatment of pharyngeal infections (Wiesner *et al.*, 1973; Karney *et al.*, 1977). As yet there is little information on the potential for oropharyngeal colonisation by β -lactamase-producing gonococci. Nevertheless, it is important to be vigilant to the possible presence in the pharynx of gonococci that are recalcitrant to treatment.

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